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NAME OF PERSON MAILING PAPER OR FEE
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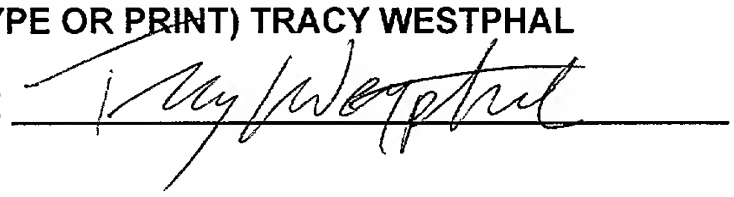
SIGNATURE 

Attorney Docket No. 0233C3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Rao et al.	Date:	November 5, 2001
Patent No.:	5,990,389	Group Art Unit:	1649
Issued	November 23, 1999	Examiner:	Kimball M.

For: High Lysine Derivatives of α-Hordothionin

Assistant Commissioner of Patents
Box REISSUE
Washington, D.C. 20231

PRELIMINARY AMENDMENT

This paper is being filed with the reissue application in the above-captioned case. The Examiner is requested to enter the following amendments and to consider the accompanying remarks. Reconsideration of the present application is respectfully requested. No new matter has been introduced.

In the Specification:

Amended figures are submitted with changes shown in red and on a separate sheet as directed in 37 CFR 1.173(b). Specifically, in Figures 3 and 4, the x-axis should be in "μg/ml" not "g/ml".

Please replace the paragraphs beginning at Column 2, line 5 - column 3, line 52 with the following rewritten paragraphs:

--α-hordothionin is a 45-amino acid protein which has been well characterized. It can be isolated from seeds of barley (*Hordeum vulgare*) and even in its native form is especially rich in arginine and lysine residues, containing 5 residues (10) of each. The amino acid sequence is as provided in SEQUENCE I.D. No. 1. It has powerful antifungal properties. Initial work to enhance the lysine content of this protein provided a high lysine derivative. However, it was impossible to predict the ultimate effect of this seemingly trivial substitution on the tertiary structure and folding of the protein, and subsequent bioassays determined that this derivative did not fold to a biologically active species in vitro. In addition, both tertiary structure and folding are critical to the stability and adequate expression of the protein in vivo, and both were absent in this compound. Therefore, further analysis and functional modeling of the wild-type compound was undertaken to determine whether substitutions could be made without disrupting biological activity. Although the crystal structure of crambin, a small protein of similar size and structure, has been reported, such crystal structures have not previously been available for hordothionin or even related compounds such as purothionin and viscotoxin. We undertook to develop such structural information.

Three-dimensional modeling of the protein led us to believe that the arginine residue at position 10 was critical to retention of the appropriate 3-dimensional structure and possible folding through hydrogen bond interactions with the C-terminal residue of the protein. A lysine substitution at that point with its shorter side chains could not hydrogen bond at the same time to both the serine residue at the 2 position and to the C-terminus while maintaining the backbone structure which we had predicted. The synthetic peptide having this substitution could not be made to fold correctly, which supported this analysis. Conservation of the arginine residue at position 10 provided a protein which folded correctly, and exhibited antifungal

activity in a bioassay. Comparison of the structure of hordothionin with that of the loosely related (48% homologous, 30% identical) protein crambin showed that thionin had a disulfide bond linking the cysteines at positions 12 and 29 which was not bridging the corresponding positions in crambin. Accordingly, replacement of the cysteine at position 12 of thionin with lysine and replacement of the cysteine at position 29 with threonine was found not to disrupt the 3-dimensional structure of the protein, as evidenced by an energy content which was determined to be indistinguishable from that of the native protein, however, substitution at position 12 did not work *in vivo*.

Further analysis of substitutions which would not alter the 3-dimensional structure of the molecule led to replacement of Asparagine-11, Glutamine-22 and Threonine-41 with lysine residues with virtually no steric hindrance. The resulting compound had 29% lysine residues. In addition, it was determined that by replacement of the serine residue at position 2 with aspartic acid, the arginine at position 10 could be replaced with lysine while permitting the needed hydrogen bonding with the C terminus. It should be appreciated that these substitutions would be effective and acceptable and could not have been predicted by examination of the linear sequence of the native thionin protein, however, substitution at position 10 did not work *in-vivo*. Other combinations of these substitutions were also made. Accordingly, this invention provides proteins having the sequence of SEQUENCE I.D. NO. 1 wherein the amino acid residues at one or more of positions 5, 11, 17, 19, 22, 30 and 41 are lysine, and the remainder of the residues at those positions are the residues at the corresponding positions in SEQUENCE I.D. No. 1. Although the native hordothionin is relatively lysine rich, a storage protein with 10% lysine residues (by number) cannot be expressed at high enough levels to obtain total protein lysine contents which are sufficient to obviate the need for lysine supplementation in poultry and swine feeds. These compounds are significantly more lysine enriched, and can be made to contain nearly thirty-percent lysine

residues. Without such enhanced lysine contents, it is impossible to eliminate the need for lysine supplementation of feeds. This invention thus also provides an important method for enhancing the lysine content of a plant cell or a plant, comprising the step of causing one or more proteins according to this invention to be expressed in the cell or plant.

Synthesis of the compounds was performed according to methods of peptide synthesis which are well known in the art and thus constitute no part of this invention. In vitro, we have synthesized the compounds on an Applied Biosystems model 431a peptide synthesizer using Fastmoc^{tm} chemistry involving hbtu [(2-(1h-benzotriazol-1-y1)-1,1,3,3-tetramethyluronium hexafluorophosphate, as published by Rao et al., *Int. J. Pep. Prot. Res.* 40:508-515 (1992). Peptides were cleaved following standard protocols and purified by reverse phase chromatography using standard methods. The amino acid sequence of each peptide was confirmed by automated Edman degradation on an Applied Biosystems 477a protein sequencer/120a pth analyzer. More preferably, however, the compounds of this invention are synthesized in vivo by bacterial or plant cells which have been transformed by insertion of an expression cassette containing a synthetic gene which when transcribed and translated yields the desired compound. Such empty expression cassettes, providing appropriate regulatory sequences for plant or bacterial expression of the desired sequence, are also well-known, and the nucleotide sequence for the synthetic gene, either RNA or DNA, can readily be derived from the amino acid sequence for the protein using standard reference texts. Preferably, such synthetic genes will employ plant-preferred codons to enhance expression of the desired protein.--

Please replace the paragraph beginning at Column 4, line 51 - column 5, line 10, with the following rewritten paragraphs:

--Synthetic DNA sequences can then be prepared which code for the appropriate sequence of amino acids, and this synthetic DNA sequence can be inserted into an appropriate plant expression cassette.

Likewise, numerous plant expression cassettes and vectors are well known in the art. By the term "expression cassette" is meant a complete set of control sequences including initiation, promoter and termination sequences which function in a plant cell when they flank a structural gene in the proper reading frame. Expression cassettes frequently and preferably contain an assortment of restriction sites suitable for cleavage and insertion of any desired structural gene. It is important that the cloned gene have a start codon in the correct reading frame for the structural sequence. In addition, the plant expression cassette preferably includes a strong constitutive promoter sequence at one end to cause the gene to be transcribed at a high frequency, and a poly-A recognition sequence at the other end for proper processing and transport of the messenger RNA. An example of such a preferred (empty) expression cassette into which the cDNA of the present invention can be inserted is the pPHI414 plasmid developed by Beach et al. of Pioneer Hi-Bred International, Inc., Johnston, IA., as disclosed in U.S. patent application Ser. No. 07/785,648, filed Oct. 31, 1991. Highly preferred plant expression cassettes will be designed to include one or more selectable marker genes, such as kanamycin resistance or herbicide tolerance genes.--

Please replace the paragraph beginning at Column 5, lines 41-42, with the following rewritten paragraph:

--Promoters that may be used in the genetic sequence include Nos, Ocs and CaMV promoters.--

Please replace the paragraph beginning at Column 6, lines 14-31, with the following rewritten paragraph:

--The isolated cloning vector will then be introduced into the plant cell using any convenient technique, including electroporation (in protoplasts), retroviruses, bombardment, and microinjection into cells from monocotyledonous or dicotyledonous plants in cell or tissue culture to provide transformed plant cells containing as foreign DNA at least one copy of the DNA sequence of the plant expression cassette. Preferably, the monocotyledonous species will be selected from maize, sorghum, wheat or rice, and the dicotyledonous species will be selected from soybean, alfalfa, rapeseed, sunflower or tomato. Using known techniques, protoplasts can be regenerated and cell or tissue culture can be regenerated to form whole fertile plants which carry and express the gene for a protein according to this invention. Accordingly, a highly preferred embodiment of the present invention is a transformed maize plant, the cells of which contain as foreign DNA at least one copy of the DNA sequence of an expression cassette of this invention.--

Please delete the sequence listing at column 9-12 and replace with the substitute sequence listing enclosed. Sequences 2-9 have been deleted. Please enter the substitute diskette in to the application. There is no new matter added.

In the Claims:

Please amend Claim 1, 2, 3 and 17 as follows:

1. (Amended) A protein having the sequence of SEQUENCE I.D. No. 1 wherein the amino acid residues at one or more of positions 5, 11, 17, 19, 22, 30 and 41 are lysine, and the remainder of the residues at those positions are wild-type.

2. (Amended) A protein according to claim 1 wherein one or more of the amino acid residues at positions 5, 11, 17, 19, 22 and 41 are lysine.

3. (Amended) A protein according to claim 2 wherein all of the amino acid residues at positions 5, 11, 17, 19, 22 and 41 are lysine.

17. (Amended) A method for killing and inhibiting plant pathogenic microorganisms which are susceptible to α-Hordothionin comprising introducing into the environment of the pathogenic microorganisms an antimicrobial amount of a protein according to claim 1.

CONCLUSION

In light of the foregoing remarks and amendments, allowance of all of the claims and reissue of this application is respectfully requested.

Respectfully submitted,

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the specification:

Paragraphs beginning at Column 2, line 5 - column 3, line 52 have been amended as follows:

α-hordothionin is a 45-amino acid protein which has been well characterized. It can be isolated from seeds of barley (*Hordeum vulgare*) and even in its native form is especially rich in arginine and lysine residues, containing 5 residues (10) of each. The amino acid sequence is as provided in SEQUENCE I.D. No. 1. It has powerful antifungal properties. Initial work to enhance the lysine content of this protein provided a high lysine derivative ~~as indicated in SEQUENCE I.D. No. 2~~. However, it was impossible to predict the ultimate effect of this seemingly trivial substitution on the tertiary structure and folding of the protein, and subsequent bioassays determined that this derivative did not fold to a biologically active species in vitro. In addition, both tertiary structure and folding are critical to the stability and adequate expression of the protein in vivo, and both were absent in this compound. Therefore, further analysis and functional modeling of the wild-type compound was undertaken to determine whether substitutions could be made without disrupting biological activity. Although the crystal structure of crambin, a small protein of similar size and structure, has been reported, such crystal structures have not previously been available for hordothionin or even related compounds such as purothionin and viscotoxin. We undertook to develop such structural information.

Three-dimensional modeling of the protein led us to believe that the arginine residue at position 10 was critical to retention of the appropriate 3-dimensional structure and possible folding through hydrogen bond interactions with the C-terminal residue of the protein. A lysine substitution at that point with its shorter side chains could not hydrogen bond at the same time to both the serine residue at the 2 position and to the C-terminus while maintaining the backbone structure which we

had predicted. The synthetic peptide having this substitution could not be made to fold correctly, which supported this analysis. Conservation of the arginine residue at position 10 provided a protein which folded correctly, [had the indicated in SEQUENCE I.D. No. 3,] and exhibited antifungal activity in a bioassay. Comparison of the structure of hordothionin with that of the loosely related (48% homologous, 30% identical) protein crambin showed that thionin had a disulfide bond linking the cysteines at positions 12 and 29 which was not bridging the corresponding positions in crambin. Accordingly, replacement of the cysteine at position 12 of thionin with lysine and replacement of the cysteine at position 29 with threonine ~~to produce a protein having the sequence indicated in SEQUENCE I.D. No. 4~~ was found not to disrupt the 3-dimensional structure of the protein, as evidenced by an energy content which was determined to be indistinguishable from that of the native protein, however, substitution at position 12 did not work *in vivo*.

Further analysis of substitutions which would not alter the 3-dimensional structure of the molecule led to replacement of Asparagine-11, Glutamine-22 and Threonine-41 with lysine residues with virtually no steric hindrance. The resulting compound had ~~the sequence indicated in SEQUENCE I.D. No.5, containing~~ 29% lysine residues. In addition, it was determined that by replacement of the serine residue at position 2 with aspartic acid, the arginine at position 10 could be replaced with lysine while permitting the needed hydrogen bonding with the C terminus~~,~~ ~~providing a compound of the sequence indicated in SEQUENCE I.D. No. 6~~. It should be appreciated that ~~that~~ these substitutions would be effective and acceptable and could not have been predicted by examination of the linear sequence of the native thionin protein, however, substitution at position 10 did not work *in-vivo*. Other combinations of these substitutions were also made~~, providing proteins having the sequences indicated in SEQUENCE I.D. No. 7 and SEQUENCE I.D. No. 8~~. Accordingly, this invention provides proteins having the sequence of SEQUENCE I.D. NO. ~~9~~ 1 wherein the amino acid residues at one or more of

positions 5, ~~10,~~ 11, ~~12,~~ 17, 19, 22, 30 and 41 are lysine, and the remainder of the residues at those positions are the residues at the corresponding positions in SEQUENCE I.D. NO. 1~~, provided that the residue at position 30 is threonine when the residue at position 12 is lysine and cysteine otherwise, and the residue at position 2 is aspartic acid when the residue at position 10 is lysine and serine otherwise~~. Although the native hordothionin is relatively lysine rich, a storage protein with 10% lysine residues (by number) cannot be expressed at high enough levels to obtain total protein lysine contents which are sufficient to obviate the need for lysine supplementation in poultry and swine feeds. These compounds are significantly more lysine enriched, and can be made to contain nearly thirty percent lysine residues. Without such enhanced lysine contents, it is impossible to eliminate the need for lysine supplementation of feeds. This invention thus also provides an important method for enhancing the lysine content of a plant cell or a plant, comprising the step of causing one or more proteins according to this invention to be expressed in the cell or plant.

Synthesis of the compounds was performed according to methods of peptide synthesis which are well known in the art and thus constitute no part of this invention. In vitro, we have synthesized the compounds on an ~~a~~Applied ~~b~~Biosystems model 431a peptide synthesizer using Fastmoc^{tm} chemistry involving hbtu ~~[~~(2-(1h-benzotriazol-1-y1)-1,1,3,3-tetramethyluronium hexafluorophosphate, as published by Rao et al., *Int. J. Pep. Prot. Res.* 40:508-515 (1992). Peptides were cleaved following standard protocols and purified by reverse phase chromatography using standard methods. The amino acid sequence of each peptide was confirmed by automated Edman degradation on an Applied Biosystems 477a protein sequencer/120a pth analyzer. More preferably, however, the compounds of this invention are synthesized in vivo by bacterial or plant cells which have been transformed by insertion of an expression cassette containing a synthetic gene which when transcribed and translated yields the desired compound. Such empty

expression cassettes, providing appropriate regulatory sequences for plant or bacterial expression of the desired sequence, are also well-known, and the nucleotide sequence for the synthetic gene, either RNA or DNA, can readily be derived from the amino acid sequence for the protein using standard reference texts. Preferably, such synthetic genes will employ plant-preferred codons to enhance expression of the desired protein."

Paragraphs beginning at Column 4, line 51 - column 5, line 10 have been amended as follows:

Synthetic ~~dna~~ DNA sequences can then be prepared which code for the appropriate sequence of amino acids, and this synthetic ~~dna~~ DNA sequence can be inserted into an appropriate plant expression cassette.

Likewise, numerous plant expression cassettes and vectors are well known in the art. By the term "expression cassette" is meant a complete set of control sequences including initiation, promoter and termination sequences which function in a plant cell when they flank a structural gene in the proper reading frame. Expression cassettes frequently and preferably contain an assortment of restriction sites suitable for cleavage and insertion of any desired structural gene. It is important that the cloned gene have a start codon in the correct reading frame for the structural sequence. In addition, the plant expression cassette preferably includes a strong constitutive promoter sequence at one end to cause the gene to be transcribed at a high frequency, and a poly-~~a~~A recognition sequence at the other end for proper processing and transport of the messenger RNA. An example of such a preferred (empty) expression cassette into which the cDNA of the present invention can be inserted is the pPHI414 plasmid developed by Beach et al. of Pioneer Hi-Bred International, Inc., Johnston, I~~d~~A., as disclosed in U.S. patent application Ser. No. 07/785,648, filed Oct. 31, 1991. Highly preferred plant

expression cassettes will be designed to include one or more selectable marker genes, such as kanamycin resistance or herbicide tolerance genes."

Paragraph beginning at Column 5, lines 41-42 has been amended as follows:

Promoters that may be used in the genetic sequence include ~~nos, ocs and camv~~ Nos, Ocs and CaMV promoters.

Paragraph beginning at Column 6, lines 14-31 has been amended as follows:

The isolated cloning vector will then be introduced into the plant cell using any convenient technique, including electroporation (in protoplasts), retroviruses, bombardment, and microinjection into cells from monocotyledonous or dicotyledonous plants in cell or tissue culture to provide transformed plant cells containing as foreign DNA at least one copy of the DNA sequence of the plant expression cassette. Preferably, the monocotyledonous species will be selected from maize, sorghum, wheat or rice, and the dicotyledonous species will be selected from soybean, alfalfa, rapeseed, sunflower or tomato. Using known techniques, protoplasts can be regenerated and cell or tissue culture can be regenerated to form whole fertile plants which carry and express the gene for a protein according to this invention. Accordingly, a highly preferred embodiment of the present invention is a transformed maize plant, the cells of which contain as foreign ~~dna~~ DNA at least one copy of the DNA sequence of an expression cassette of this invention.

In the claims:

Claims 1, 2, 3 and 17 have been amended as follows:

1. (Amended) A protein having the sequence of SEQUENCE ~~I~~ I.D. No. 1 wherein the amino acid residues at one or more of positions 5, ~~10~~, 11, ~~12~~, 17, 19, 22, 30 and 41 are lysine, and the remainder of the residues at those positions are ~~the residues at the corresponding positions in SEQUENCE I.D. No. 1~~ wild-type.

2. (Amended) A protein according to claim 1 wherein one or more of the amino acid residues at positions 5, 11, ~~12~~, 17, 19, 22 and 41 are lysine.

3. (Amended) A protein according to claim 2 wherein all of the amino acid residues at positions 5, 11, ~~12~~, 17, 19, 22 and 41 are lysine.

17. (Amended) A method for killing and inhibiting plant pathogenic microorganisms which are susceptible to ~~a~~ α-Hordothionin comprising introducing into the environment of the pathogenic microorganisms an antimicrobial amount of a protein according to claim 1.

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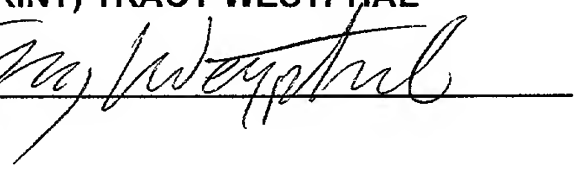
SIGNATURE 

Attorney Docket No. 0233C3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Rao et al.	Date:	November 5, 2001
Patent No.:	5,990,389	Group Art Unit:	1649
Issued	November 23, 1999	Examiner:	Kimball, M.

For: High Lysine Derivatives of α-Hordothionin

Assistant Commissioner of Patents
Box REISSUE
Washington, D.C. 20231

STATEMENT OF STATUS AND SUPPORT UNDER 37 CFR 1.173(c)

This paper is being filed with the reissue application in the above-captioned case.

Status of the Claims:

Claims 1-21 are pending. Claims 1-3, and 17 have been amended and no new claims have been added.

In the Specification:

Amended Figures 3 and 4 are supported by Figures 3 and 4 in W094/16078 (enclosed) where the legend for the x-axis is in μg/ml.

Support for deleting residue 10 in claim 1, is found in the instant specification, column 2, lines 28-40 where it states:

> "Three-dimensional modeling of the protein led us to believe that the arginine residue at position 10 was critical to retention of the appropriate 3-dimensional structure and possible folding through hydrogen bond interactions with the C-terminal residue of the protein. A lysine substitution at that point with its shorter side chains could not hydrogen bond at the same time to both the serine residue at the 2 position and to the C-terminus while maintaining the backbone structure which we had predicted. The synthetic peptide having this substitution could not be made to fold correctly, which supported this analysis. Conservation of the arginine residue at position 10 provided a protein which folded correctly, and exhibited antifungal activity in a bioassay."

Support for deleting residue 12 in claims 1-3, is found in the instant specification, column 2, lines 41-52 where it states:

> "Comparison of the structure of hordothionin with that of the loosely related (48% homologous, 30% identical) protein crambin showed that thionin had a disulfide bond linking the cysteines at positions 12 and 29 which was not bridging the corresponding positions in crambin. Accordingly, replacement of the cysteine at position 12 of thionin with lysine and replacement of the cysteine at position 29 with threonine [to produce a protein having the sequence indicated in SEQUENCE I.D. No. 4] was found not to disrupt the 3-dimensional structure of the protein, as evidenced by an energy content which was determined to be indistinguishable from that of the native protein, however substitution at position 12 did not work in-vivo."

Support for amending a-Hordothionin to α-Hordothionin in claim 17, is found in the instant specification in the title and in column 2, line 5, and is to correct a typographical error.

Deleting [that], adding and in line 64 and 65, column 2, capitalizing Applied Biosystems in line 31, column 3, changing a bracket to a parenthesis in line 32, column 3, capitalizing DNA in lines 51 and 53, column 4, and in column 6, lines 19 and 30, capitalizing poly-A in line 1, column 5, capitalizing Nos, Ocs and CaMV in line 42 and amending a-Hordothionin to α-Hordothionin in claim 17 is to correct typographical errors, grammatical errors or errors in usage or capitalization of scientific language.

References to SEQUENCE I.D. NOS. 2 -9 are deleted in order to correct errors in these sequences due to both numbering confusion with crambin and to the removal of positions 10 and 12 from the claims. Also, references are deleted to reflect the claiming of only SEQUENCE I.D. NO. 1.

CONCLUSION

In light of the foregoing remarks and amendments, allowance of all of the claims and reissue of this application is respectfully requested.

Respectfully submitted,

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Registration No. 44,041

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PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

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(43) International Publication Date: 21 July 1994 (21.07.94)

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08/003,885 13 January 1993 (13.01.93) US

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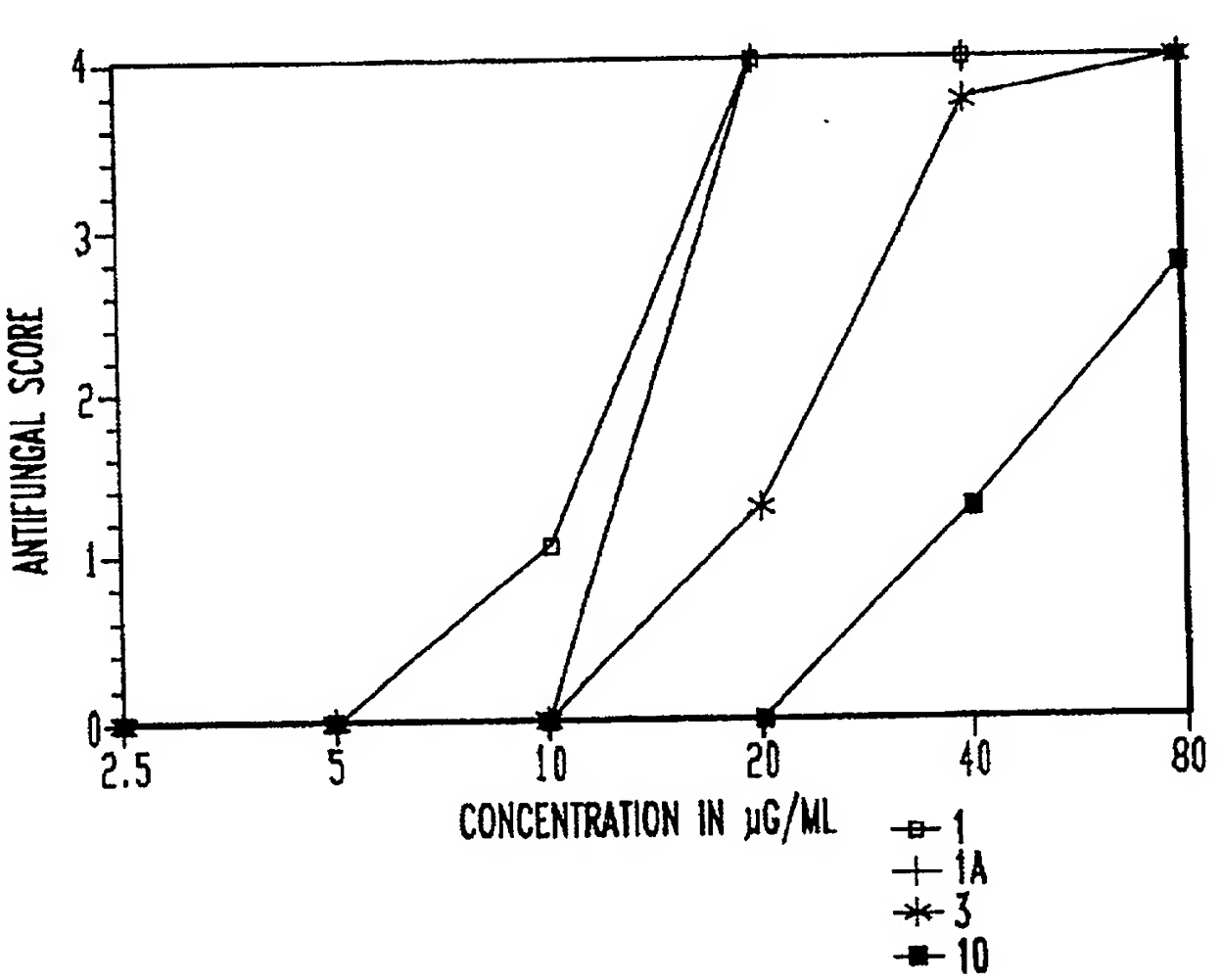
(74) Agents: ROTH, Michael, J. et al.; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

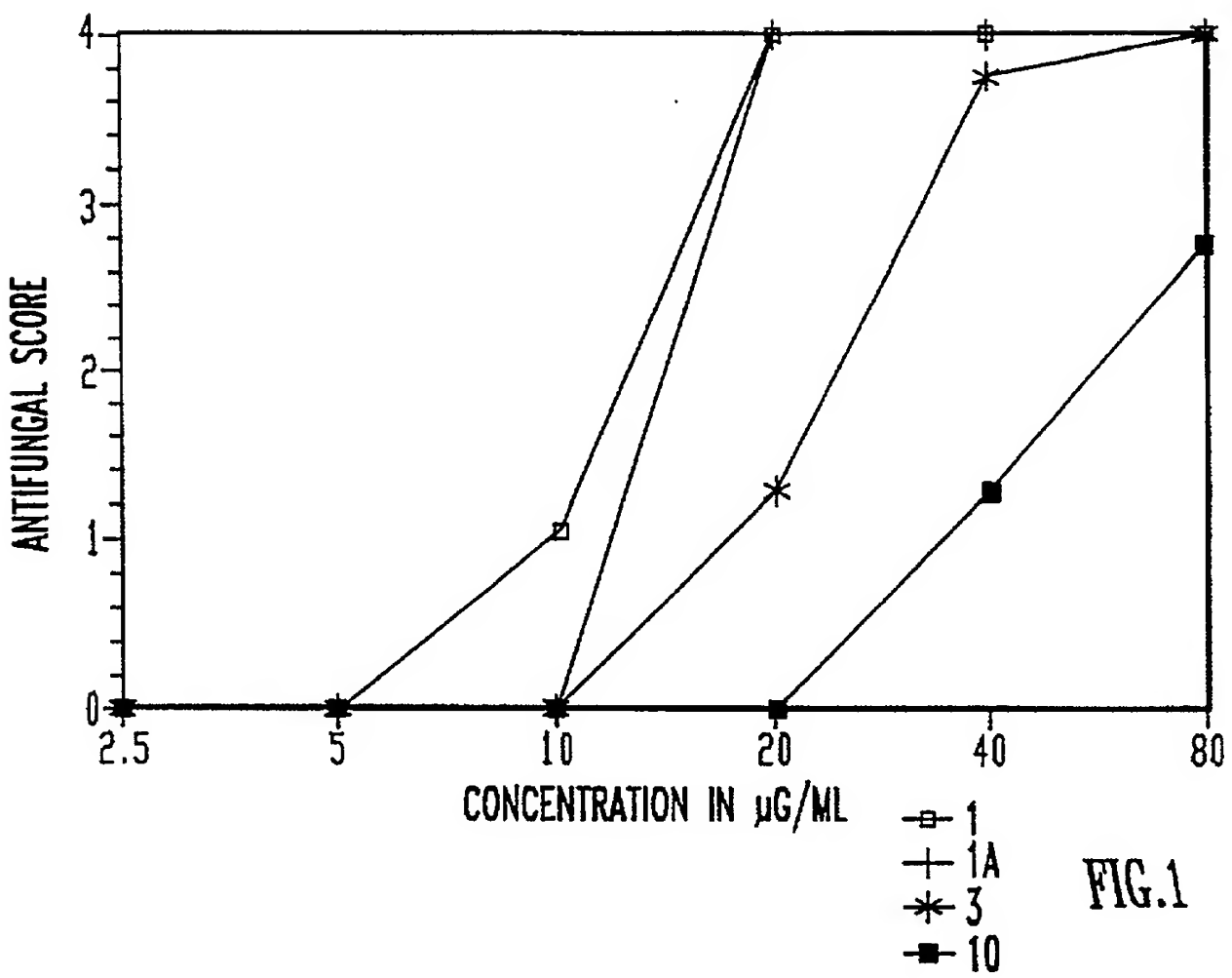
Published
Without international search report and to be republished upon receipt of that report.

(54) Title: HIGH LYSINE DERIVATIVES OF ALPHA-HORDOTHIONIN



(57) Abstract

Derivatives of α-hordothionin made by position-specific substitution with lysine residues provide lysine enrichment while retaining the antifungal activity of the parent compound.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1
2
3
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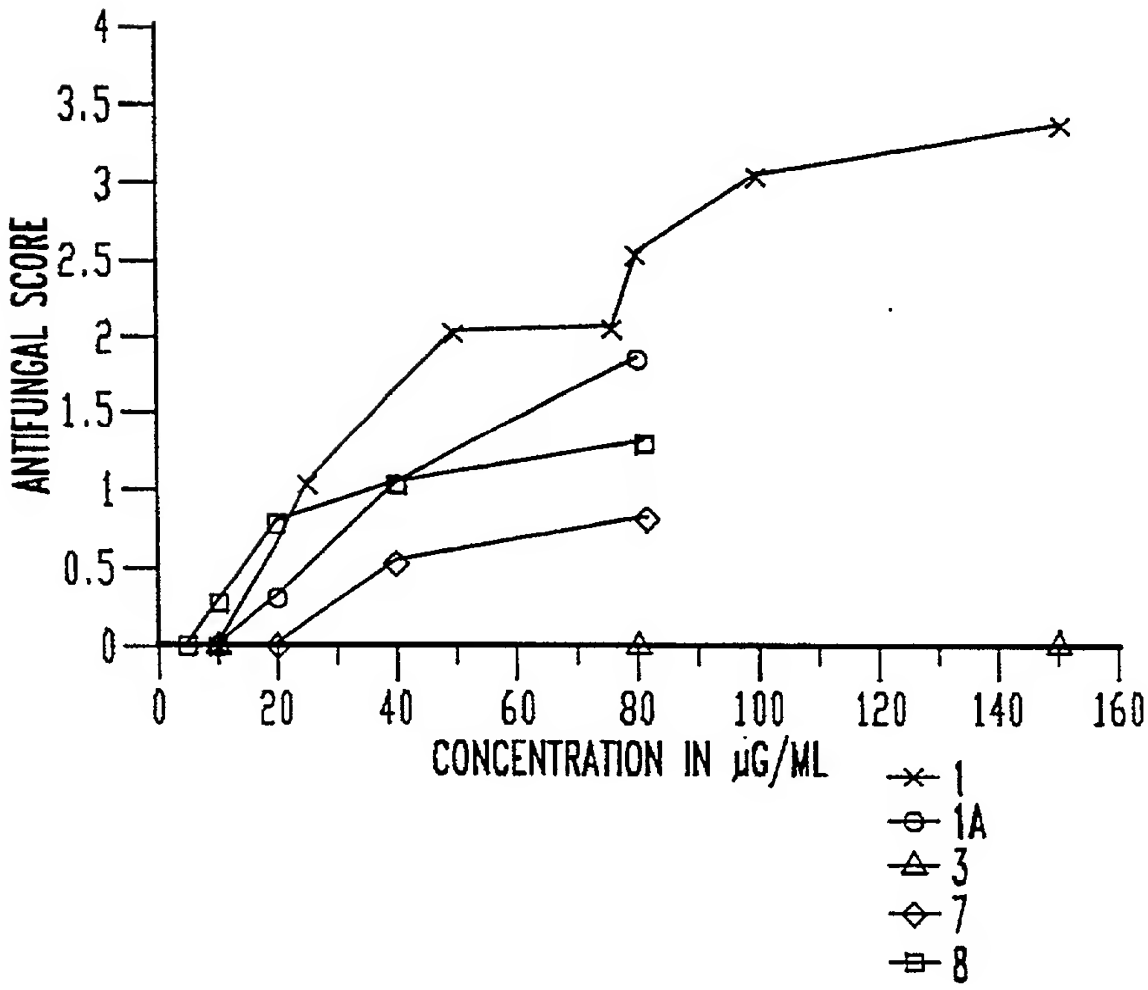
2.5
5
10
20
40
80
CONCENTRATION IN µG/ML
1
1A
3
10

FIG.1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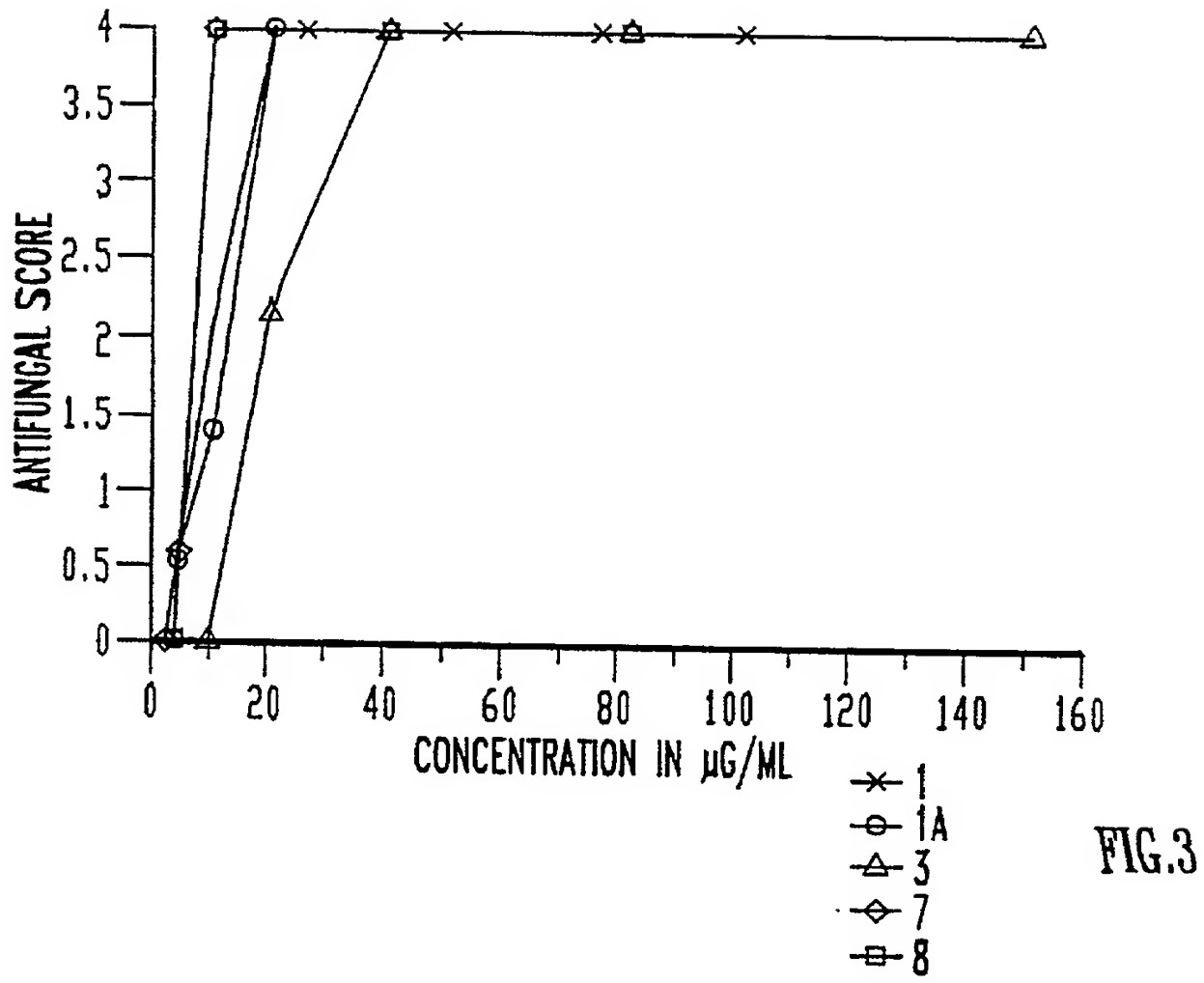
1A
3
7
8

FIG.2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1
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80
100
120
140
160
CONCENTRATION IN µG/ML
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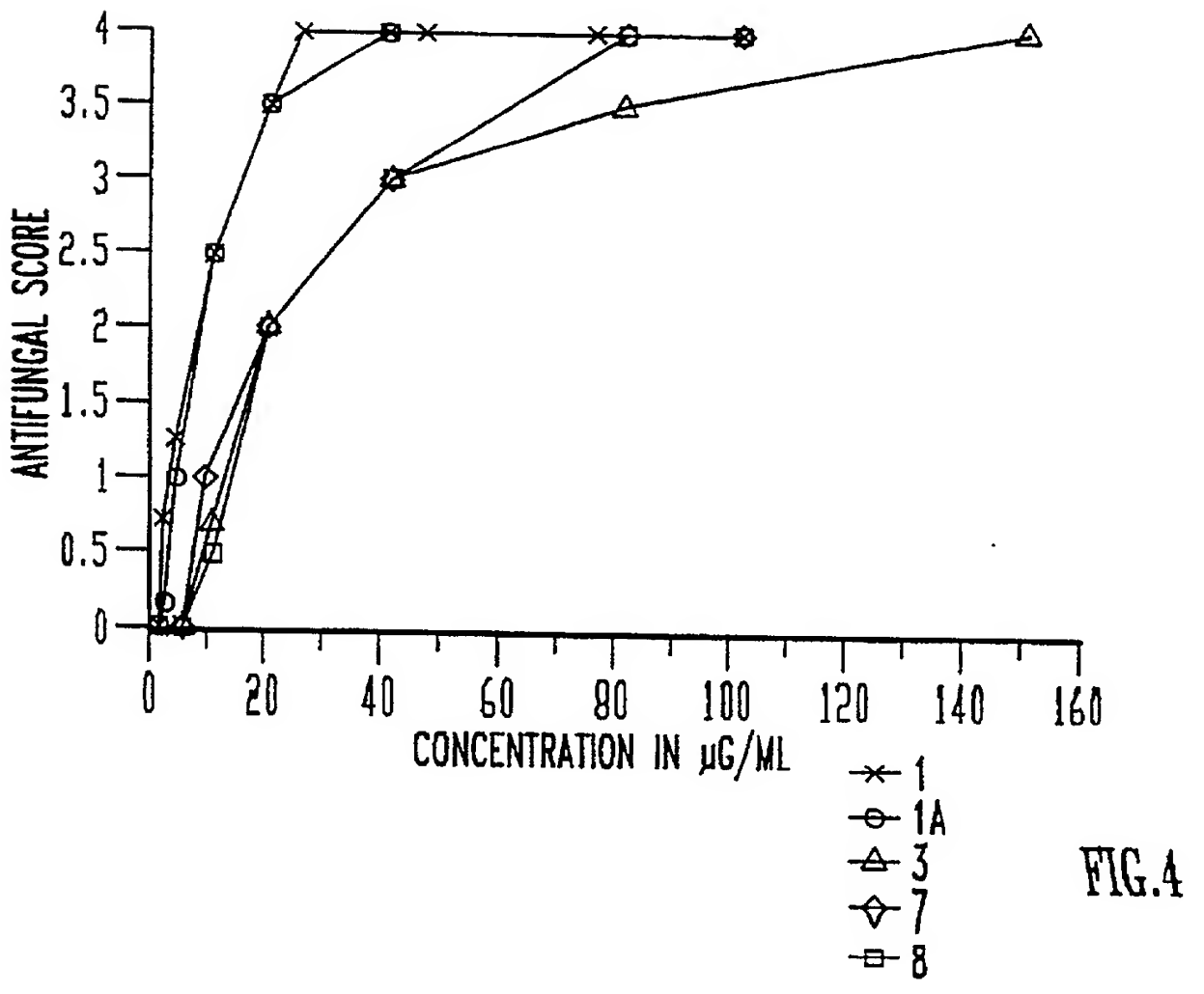
1A
3
7
8

FIG.3


ANTIFUNGAL SCORE
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3.5
3
2.5
2
1.5
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0.5
0
0
20
40
60
80
100
120
140
160
CONCENTRATION IN µG/ML
1
1A
3
7
8

FIG.4